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A Substance Inducing the Loss of Premature Embryos From Ovigerous Crabs

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Abstract. The embryos of an estuarine terrestrial crab (akate-gani; *Sesarma haematocheir*) are attached by a funiculus to ovigerous hairs on the maternal pleopods, and are ventilated by the female until hatching occurs. When females were kept in a small quantity of diluted seawater (about 10‰), hatching and larval release occurred in all cases. In contrast, when the medium was *hatch water* (*i.e.*, the filtered medium into which larvae had been released), most ovigerous females liberated their embryos prematurely, but hatching did not occur. The egg masses (cluster of embryos) carried by these females were not released as usual, but were gradually extruded from the brooding chamber, and within a few days all had dropped to the bottom of the beaker. No morphological changes were found on the outer egg membrane, the funiculus, or the coat investing the ovigerous hairs of females kept in hatch water. But the ovigerous hairs did slip easily out of the *coat*, and this caused the extrusion of the egg masses. The active factor—called *incubation disrupting substance*—was stable with freezing, but heat-labile. In normal females (*i.e.*, those not treated with hatch water), broken egg cases and funiculi remain for a time after hatching with the coat on the ovigerous hairs, but they are gone by the morning after hatching. So the secretion of this incubation-disrupting substance may participate in cleaning the ovigerous hairs of old investing coats and funiculi after larval release, thus preparing for the attachment of the next clutch of embryos. In addition, this substance may play a role in hatching.

Introduction

The newly oviposited eggs of decapod crustaceans, except nonbrooding peneid shrimps, are wrapped in a thick transparent outer membrane and clustered on pleopod setae beneath the folded abdomen of the female (Herrick, 1895; Yonge, 1937, 1946, 1955; Cheung, 1966; Fisher and Clark, 1983; Goudeau and Lachaise, 1983). The embryos are attached to the pleopod setae by a stalk: the funiculus. While attached to the ovigerous hairs by this stalk, the embryos are ventilated by the movement of pleopods of the female until the thick outer membrane breaks, indicating the beginning of hatching. The present study is concerned, in general, with hatching in an estuarine terrestrial crab; it is focused on an active substance that is released *outside* of the egg membrane at the time of hatching.

Hatching enzyme is among the important substances that are discharged upon hatching in many groups of animals. In the teleost *Oryzias latipes*, the enzyme, which is secreted by the embryo, digests the egg membrane, allowing the embryo to emerge (*e.g.*, Ishida, 1944; Yamagami, 1972). This teleost enzyme is actually a mixture of two kinds of proteases that act cooperatively on the inner layer of the egg case (Yasumasu *et al.*, 1989a, b). Embryos of the toad *Xenopus laevis* also secrete a proteolytic enzyme (from the frontal region of the embryos) that digests the fertilization membrane (Carroll and Hedrick, 1974). Katagiri (1975) also demonstrated a hatching enzyme of 55–60 kDa that digests the whole egg case in the frog *Rana chensinensis*. Similarly, sea urchin embryos at the blastula stage (about 10 h after fertilization) secrete a proteolytic enzyme that dissolves the fertilization membrane. Recently, Lapage and Gache (1989) purified the enzyme, a glycoprotein of 51, 52, or 57 kDa that autolyzes to an inactive form of 30 kDa.

Among crustaceans, *adult* barnacles (*Balanus*) release a substance that stimulates the hatching of nauplius larvae (Crisp and Spencer, 1958). If the barnacles were fed, the factor was secreted within 1 or 2 days and the embryos

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hatched; if the barnacles were starved, hatching did not occur. Because the factor could be extracted from the tissues of starved animals in the laboratory, as well as those fed under natural conditions, it may be produced by the barnacle's own metabolism, and not related to a particular food source (Crisp and Spencer, 1958). An active factor extracted and purified from adult barnacle tissues contains a prostaglandin-like compound that stimulates embryonic muscle (Clare *et al.*, 1982).

In the brine shrimp *Artemia salina*, glycerol is highly concentrated in dormant cysts, and then rapidly disappears (Clegg, 1962). When the embryos emerge from their egg cases, glycerol is released into the medium (Clegg, 1964).

Embryos of the terrestrial crab *Sesarma haematocheir* hatch on land around the time of nocturnal high water. Hatching occurs in a very short time, and the timing is highly synchronized among embryos (Saigusa, 1992, 1993). As soon as hatching is complete, the crabs enter the water and release the hatched larvae by making vigorous fanning movements of the abdomen (Saigusa, 1982). The embryos of ovigerous females that were collected from the field and kept in the laboratory did hatch simultaneously. This paper first demonstrates that the filtered water into which zoea-larvae had hatched (*i.e., hatch water*) contains a substance that disrupts the incubation of *other* females. Next, microscopic studies are described that indicate the cause of the liberation of premature embryos from the brooding chamber.

Materials and Methods

Animal handling

Experimental animals were ovigerous females of the terrestrial red-handed crab (akate-gani), *Sesarma haematocheir*, collected from the thicket along a small estuary at Kasaoka, Okayama Prefecture, Japan. (For details of the habitat and the chronology of larval release, see Saigusa, 1982.) Collections were made on 19 August and 2, 4, 9, 14, 15, and 16 September 1991. The crabs were quickly brought into the experimental rooms, where they were kept in plastic containers (70 cm long, 40 cm wide, and 25 cm high) containing shallow water (*ca.* 1 cm deep) and hiding spaces. The water was renewed whenever one or more females released their larvae in the container, and otherwise at intervals of 3–5 days. The crabs were fed every few days.

Light and temperature were controlled in the experimental rooms. A 15-h light and 9-h dark photoperiod, a phase that is much the same as that in the field (light-on at 0500 and light-off at 2000), was employed. Temperature was held constant at 23 ± 1°C. Under this light condition, larval release by females occurs about the time of nocturnal high tides in the field.

Preparation of hatch water

Eggs of *S. haematocheir* are dark brown at the early stages of embryonic development, but become a brownish green (possibly because of yolk consumption) as hatching approaches. Females carrying brownish-green eggs (*i.e.*, embryos that should hatch within a few days) were selected, removed from the plastic containers, and placed individually in glass or plastic beakers (8.5 cm in diameter, 12 cm in height) containing 30 ml of 10‰ seawater. This medium is prepared from natural seawater, which is boiled for 5–10 min, cooled, and then diluted with distilled water; the solution is aerated for at least one day before use. The medium was renewed at intervals of 1–2 days. Under these conditions, all of the females continued to carry eggs until hatching (Table I, top).

Hatching of *S. haematocheir* is highly synchronized; all of the embryos may hatch within about 5–30 min in the laboratory (*e.g.*, see Saigusa, 1993). When hatching was completed, and the female had released all of her larvae into the medium by vigorous abdominal fanning, she was removed, and the medium was filtered through nylon mesh or filter paper (*filtrate 1* in Fig. 1) which removed the zoeas. As shown in Table I, 23 samples of *filtrate 1* were prepared immediately after the larval release

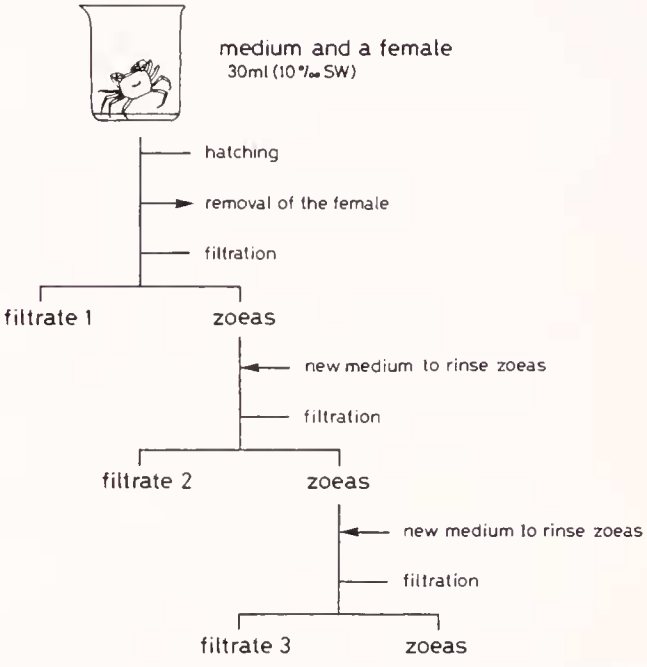


Figure 1. Collection of hatch water. Each medium contained 30 ml of 10‰ seawater. See the text for details.

(*i.e.*, within 30 min); the rest (22 samples) were prepared after a while (*i.e.*, more than 30 min after the release, but before 10:00 on the next morning following release).

The filtered zoeas were immediately immersed in new medium, and this medium was also filtered (*filtrate 2*). Among *filtrate 2*, nine samples were generated immediately after the preparation of *filtrate 1*, and then the zoeas were rinsed and filtered once more (*filtrate 3*) (Fig. 1). The rest of *filtrate 2* (32 samples) was generated within a few hours after the preparation of *filtrate 1*. Because larval release occurs at night, these procedures were usually carried out under illumination provided by a small hand-held light.

Assay of the activity of incubation-disrupting substance

Ovigerous females, that had not been used to collect the hatch water, were selected randomly from the stock containers and placed individually in beakers containing hatch water (*filtrates 1, 2,* or *3*). The activity of incubation-disrupting substance in each filtrate was evaluated in terms of the following three grades: (+), a portion of the egg mass protrudes from the brooding space; (++), the egg-mass protrusion progresses further, and some embryos are scattered at the bottom of the beaker; (+++), all, or almost all, of the egg mass has slipped out of the brooding space. Observations were finished 4 or 5 days after the female had been placed in each beaker. To prevent contamination of the medium by feces and foods, the females were not fed during the experiment. With time, however, they often ate their own embryos, either those released in the beaker or those squeezed out of the brooding chamber. But females never eat their embryos in normal brooding. For these reasons, the effect of test solutions was judged within 4–5 days.

Observations with a scanning electron microscope

Embryos attached to the pleopod setae, as well as those released in the hatch water, were collected and fixed with 5% formalin in distilled water for 2 or 3 days. They were then washed a few times in 50% ethanol, then dehydrated through a graded series of ethanol concentrations and dried in a critical point apparatus (Hitachi HCP-1). The samples were mounted on metallic stubs with a piece of double-sided stick tape, plated with gold, and observed with a scanning electron microscope (JEOL JSM-T300).

Boiling and freezing of the test samples

A sample of *filtrate 1*, obtained from 15 females that had just released their larvae, was boiled for 5 min. After it had cooled, the solution was divided among 15 beakers (30 ml each), and an incubating female was put into each. Another sample of hatch water (*filtrate 1*), obtained similarly from seven specimens, was frozen at −80°C for 8 days, and then thawed at room temperature. This sample was divided among seven beakers, and an ovigerous female was again placed in each.

Results

Egg attachment to pleopod setae

A female of *Sesarma haematocheir* has four pairs of abdominal appendages, each of which bears one plumose and one non-plumose seta (Fig. 2A). Many fine hairs (*ovigerous hairs*) are arranged in whorls along the non-plumose seta (Fig. 2B). Fertilized eggs are attached to these hairs by a *funiculus* (Fig. 2C, D). When the ovigerous hair was removed with fine forceps, the *coat* that had been wrapped around the hair was broken but remained attached to several funiculi (Fig. 2E). This observation suggests that the investment coat is formed of the same material as the funiculus (Fig. 2E, F). The morphological features shown in Figure 2D, E, and F suggest that this material also constitutes the outer egg membrane, or at least covers the surface of this membrane.

Protrusion and dropping of egg masses from an incubating female

As shown in Figure 3A, an incubating female carries her clutch in the brooding space between the thorax and the flexed abdomen. Premature embryos never slipped out of the brooding space when the females were in 10‰ seawater, and hatching occurred in all cases (Table I). At the completion of hatching, these females released the hatched larvae into the medium with vigorous fanning movements of the abdomen.

Soon after ovigerous females were placed in hatch water, a mass of premature embryos protruded from the brooding chamber, and then dropped into the beaker. This process is shown in Figure 3B–E′. Figure 3B shows a portion of the incubating egg mass starting to protrude from the brooding space (+). In Figure 3C and D, the embryo-protrusion has proceeded further (++). These premature embryos were often released by the females in association with abdominal fanning behavior and were scattered at the bottom of the beaker. In Figures 3E and 3E′, the embryos have all (or almost all) dropped from the brooding chamber (+++). A remarkable feature of this phenomenon is that, from the beginning of embryo-protrusion to the time that all of the egg masses have slipped out of the female takes only a few days. As shown in Table I, 37 of 45 females (82%) treated with hatch water (*filtrate 1*) released their egg masses into the beaker. Clearly, the active substance that disrupts incubation is contained in hatch water.

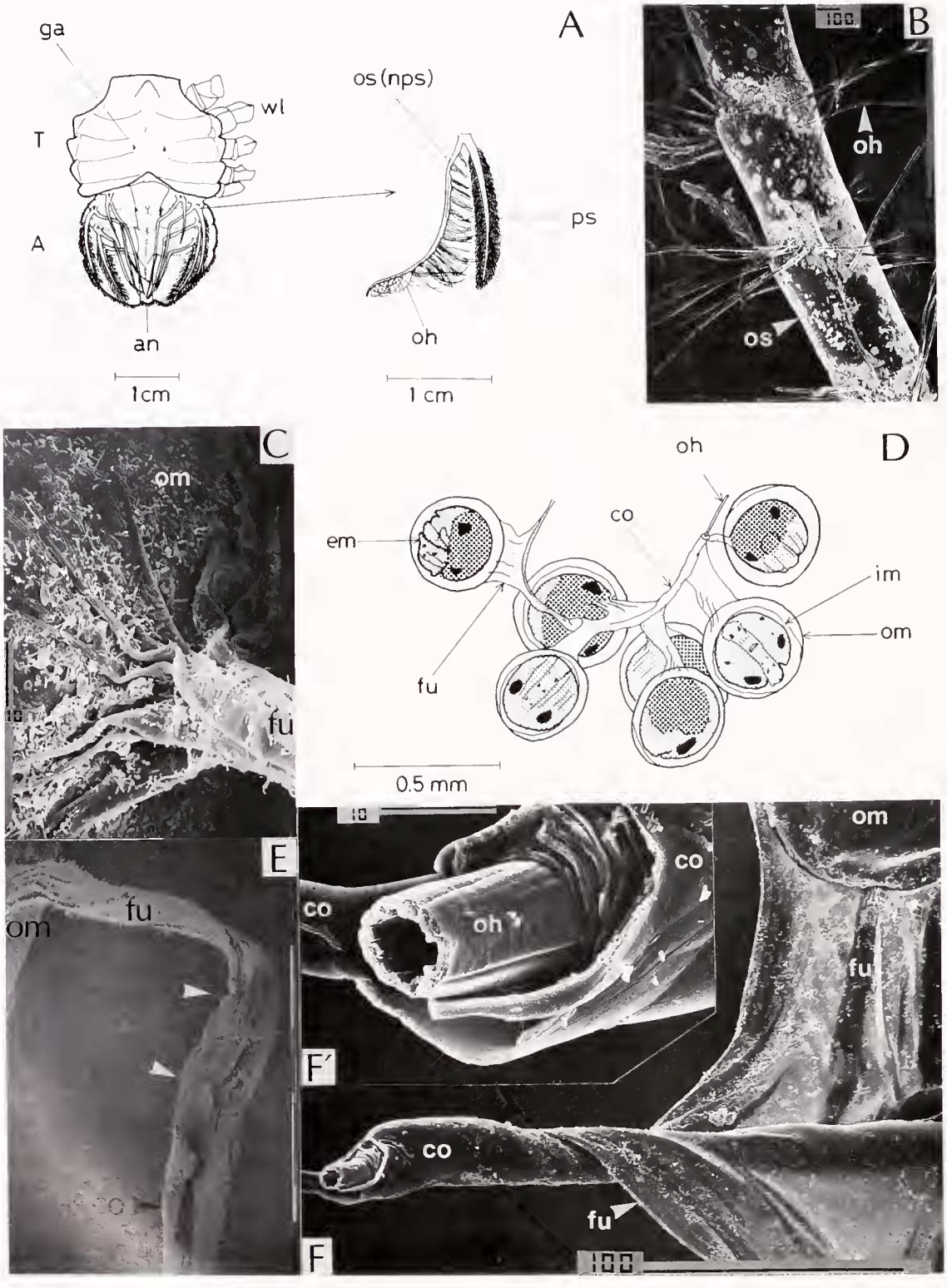


Figure 2. The pleopod setae and attachment of eggs. (A—left) Thorax (T) and abdomen (A) of a *Sesarma haematocheir* female. wl: walking leg; an: anus; ga: genital aperture. (A—right) The second abdominal appendage showing plumose and non-plumose setae. ps: plumose seta; os(nps): non-plumose seta; oh: ovigerous hair. (B) Ovigerous hairs on the non-plumose seta. Scale = 100 μm. (C) The outer egg membrane (om) and funiculus (fu). Scale = 10 μm. (D) Embryos attached to an ovigerous hair by funiculus. This specimen was fixed with 5% formalin and observed in 50% ethanol. em: embryo; fu: funiculus; co: the coat investing an ovigerous hair; oh: ovigerous hair; im: inner (or lining) membrane; om: outer thick membrane. In living

The activity of any sample of hatch water (*filtrate 1*) seems to vary with the number of zoea-larvae released into the medium. When the hatch water was derived from medium into which a large number of zoea had been released, almost all of the females dropped their embryos within 1 day after contact. But hatch water prepared from a medium containing only a small number of larvae, induced this phenomenon 1–3 days later.

Four females, though treated with hatch water (*filtrate 1*), showed no sign of egg mass protrusion (Table I). This negative result, however, could have been due to an insufficient concentration of active substance. When the unresponsive females were isolated after the experiment in a new, unoccupied container, they finally dropped their egg masses some days later (unpub. obs.). In four other females (Table I), normal hatching and larval release occurred before egg mass protrusion could begin. Presumably, these ovigerous females had been collected just prior to hatching, which simply occurred on schedule, uninfluenced by the incubation-disrupting substance reported here.

The 15 samples of boiled hatch water had no effect on incubation: no egg masses protruded from the abdomen (Table I), so the incubation of the test females was clearly maintained. In contrast, the egg masses protruded and dropped as usual when seven females were exposed to hatch water that had been frozen and thawed (Table I). Incubation-disrupting substance is thus stable to freezing, but is heat-labile.

To determine whether the active substance is released in association with larval hatching or is generated by the larvae after the release, the effects of *filtrates 2* and *3* were compared with that of *filtrate 1* (Table I). The activity was markedly decreased in *filtrate 2*, and no effect was detected in *filtrate 3*. Because *filtrate 1* contains most of the incubation-disrupting activity, I speculate that the responsible factor is all released to the medium when the egg membrane opens at the time of hatching.

Microscopic observations of the egg masses liberated prematurely from the female

Clusters of embryos shed into the incubation medium were observed under a stereomicroscope (Fig. 4A). The surface of the embryos (*i.e.*, outer egg membrane) and the funiculus were not different morphologically from the eggs attached to intact females. The embryos were obviously alive (Fig. 4A), and the funiculus remained attached to the coat investing each ovigerous hair. But a clear difference was observed *in the coat* investing ovigerous hairs. Egg masses incubated by untreated females were attached tightly to the ovigerous hairs (Fig. 2F and F′), whereas those dropped in hatch water always lacked an ovigerous hair in the twig of the investment coat (Fig. 4B). Premature embryo release is thus due to the investment coat separating from the ovigerous hairs, and not the funiculus detaching from the coat.

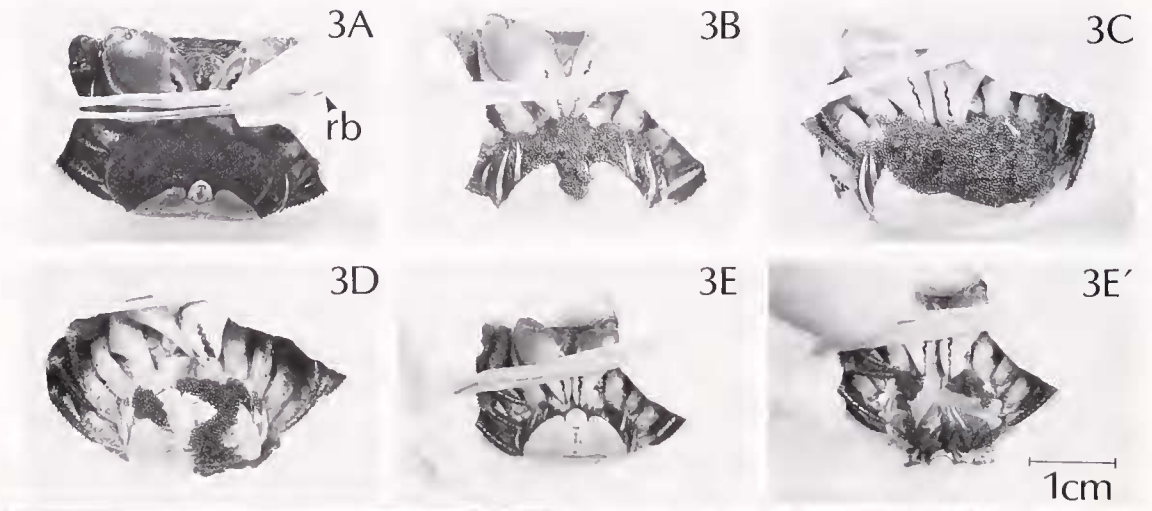
When hatch-water-induced protrusion of egg masses was in progress (+ or ++; Fig. 5A), groups of embryos would easily slip off the ovigerous hairs when gently pulled with a fine forceps (Fig. 5B). These embryos were always accompanied by the funiculus and coat. In contrast, when the egg mass of a control female was pulled, the ovigerous hairs were broken away from the seta (not shown).

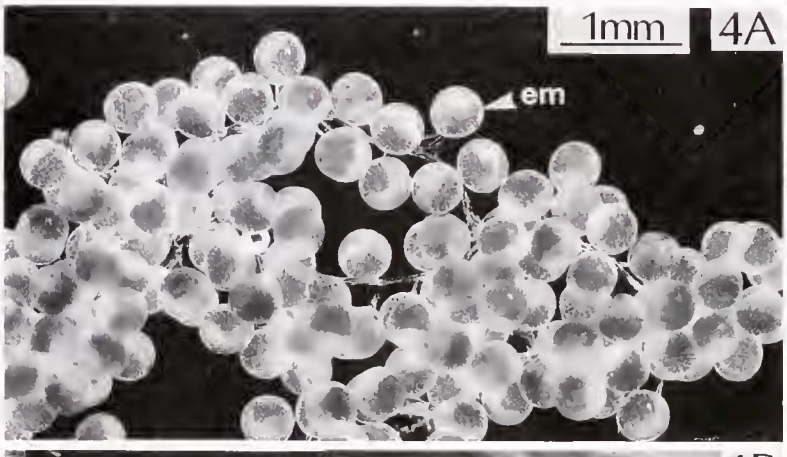
Discussion

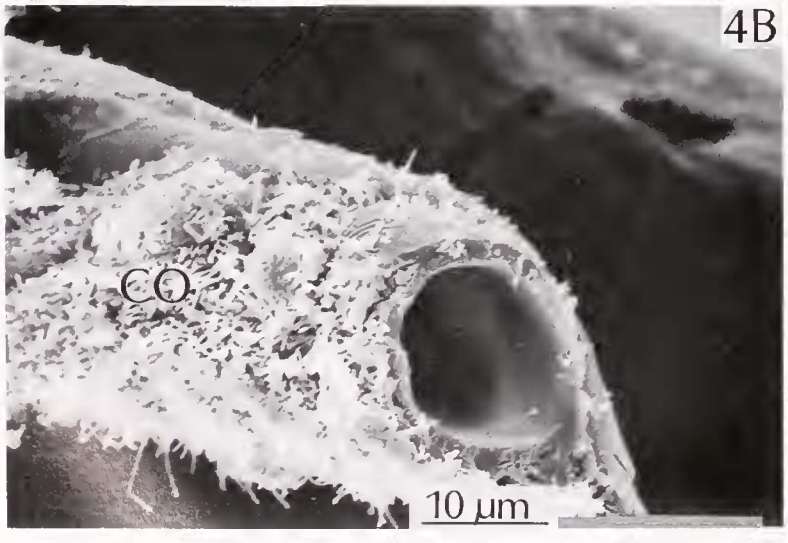
The embryos of most decapod crustaceans are incubated by the females until hatching occurs. So it may be natural that a portion of the egg cluster is lost from the female during embryonic development. For example, some embryos may be dropped when the female cleans the clutch using her chelae. Laboratory-maintained females of the lobsters *Homarus americanus* and *H. gammarus* often lose a large percentage of embryos because the funiculus has been improperly formed (Talbot and Harper, 1984). In *Sesarma haematocheir*, predation on egg masses by the maggots of a parasitic fly can be the main extrinsic factor for the loss of embryos in the field (unpub. obs.). But the phenomenon reported in the present study is quite different from such types of egg loss.

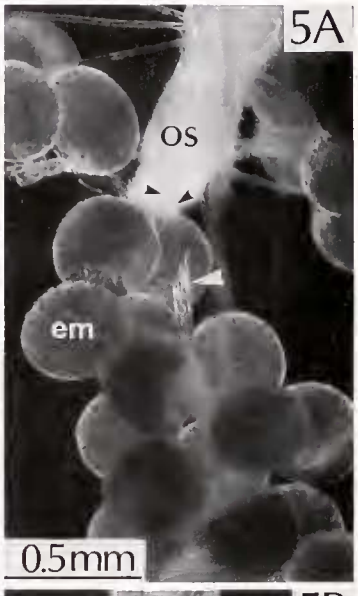
Disruption of incubation is certainly an unusual phenomenon for ovigerous females; but this phenomenon is obviously adaptive for females that have released their larvae. Observations of the female abdomen just after larval release (Saigusa, 1992, 1993) demonstrated that the broken egg case (outer egg membrane), funiculus, and the coat still remain attached to the ovigerous hairs (see also Fig. 6A). A similar event occurs in the lobsters *H. americanus* and *H. gammarus;* egg cases and stalks remain attached on the ovigerous hairs, and they seem to be cast

Figure 2. (*Continued*) specimens, the embryo completely fills, and is pressed against the egg case, but in the fixed samples, the outer membrane swells, revealing an inner membrane or a membrane lining the egg case (for egg membranes, see Saigusa, 1992). (E) The coat from which an ovigerous hair was removed with a fine forceps. White arrow heads show the region where the funiculus (fu) branches out of the investment coat. Scale = 100 μm. (F) The coat wrapping an ovigerous hair. Scale = 100 μm. Upper left (F′): Magnification of the portion where this hair penetrates the coat. Scale = 10 μm.









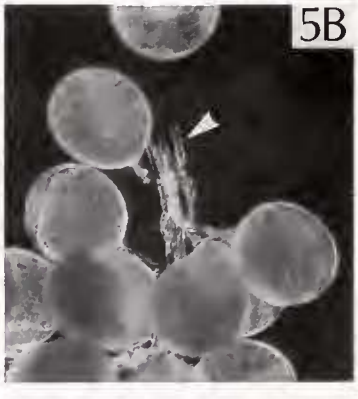


Figure 3. Premature egg-release from a female. (A) A normal clutch incubated by a female. rb: a rubber band to fix the female's body and legs. (B) A female in which egg-release from the brooding chamber is beginning (+). (C and D) Females in which protrusion of egg mass has proceeded further (++). (E) A female from which almost all eggs were released (+++). (E′) The inside of the brooding chamber in this female. Although some eggs still remained in the chamber, they have already been detached from the ovigerous hairs.

Figure 4. Morphology of premature eggs liberated from the female. (A) Embryos (em) extruded from the brooding chamber and dropped to the bottom of the beaker. These embryos (at early developmental stage) are all alive. The coats that invested the ovigerous hairs are seen to be in place. (B) An ovigerous hair is absent from its investment coat (co). An ovigerous hair itself is also hollow, but its appearance is clearly different from that of the coat (compare with Fig. 2F′).

Table I

Liberation of premature embryos induced by the hatch water

Medium	Number	Activity of filtrate (+)	(++)	(+++)	Maintenance of incubation	Hatching and larval release
Fresh medium (10‰ SW)	82	0	0	0	(0)	82
Filtrate 1						
Generated within 30 min after hatching	23	0	0	21	1	1
Generated more than 30 min after hatching	22	0	0	16	3	3
Boiling	15	0	0	0	14	1
Freezing and thawing	7	1	0	6	0	0
Filtrate 2						
Generated within 1 h after hatching	9	0	0	0	8	1
Generated for 3–10 h after hatching	32	1	0	2	23	6
Filtrate 3						
Generated within 1 h after hatching	9	0	0	0	9	0

off from the ovigerous hairs at the next molt (Goudeau *et al.*, 1987).

In contrast to the lobster, the remnants on the ovigerous hairs of *S. haematocheir* completely disappear during the night of the larval release, and the next clutch is produced within a few days. After larval release is complete, females unfold their abdomen and pluck the empty egg cases and funiculi to eat; indeed, some of the remnants are probably disposed of in this way. But it is not conceivable that a purely mechanical plucking behavior can completely clear ovigerous hairs and setae. I conclude that the incubation-disrupting substance, as reported in this paper, causes the investment coat to slip off the ovigerous hair, facilitating post-release remnant-eating of the female, and releasing the remnants that are not eaten. Because the ovigerous hairs completely clean off by the morning following larval release (compare Fig. 6A and B), incubation-disrupting substance may also make it easier for the female crab to get the debris off her ovigerous hairs *without damaging or breaking the hairs* which, after all, must be in good condition to receive the new clutch of eggs. In brief, the incubation-disrupting substance clears the ovigerous hairs for the next clutch of eggs.

What mechanisms cause the investment coat to slip from ovigerous hairs? The exact mechanisms of egg attachment to the ovigerous hairs and the source of the attachment stalk have been controversial for a number of years (Yonge, 1937, 1946, 1955; Cheung, 1966; Fisher and Clark, 1983). Goudeau and Lachaise (1983) showed that the funiculus of the American lobster *Homarus* folds around an ovigerous hair, forming several turns, and that for the innermost coiling turn, the stalk wall adheres tightly to the hair surface, although no adhesive substance is detectable. In *S. haematocheir,* the funiculus extends to the coat wrapping an ovigerous hair, which suggests that the same material forms both the stalk and the coat (Fig. 2E, F). Furthermore, this material might make up the outer egg membrane (at least the surface of the membrane), as well (Fig. 2C). Because the coat dropped from the female shows no obvious morphological changes (Figs. 4A, B), then the egg masses must be liberated, either by a deformation of the ovigerous hairs or by a separation of the coat in some unknown way from the hairs. But the deformation of the ovigerous hairs must be detrimental to the hairs because a new clutch has to be attached to them.

Embryos of the estuarine crab *Rhithropanopeus harrisii* can be detached by bovine or porcine trypsin, and are released into the medium by the pumping movements of the females. Some of them prematurely hatched as im-

Figure 5. Detachment of the egg masses from the ovigerous hairs (living specimen). (A) Two ovigerous hairs (see the black arrow heads) and the investing coat that begins to slip out of these hairs. A white arrow head indicates the edge of the investment coat. em: embryo; os: ovigerous seta. (B) The end (a white arrow head) of a section of coat that had slipped off its enclosed ovigerous hairs.

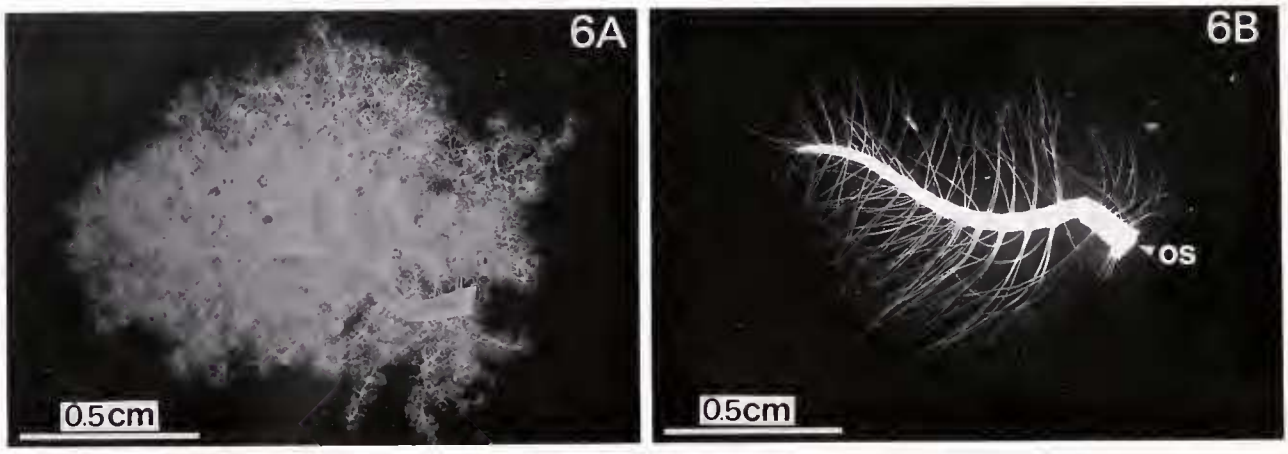


Figure 6. Ovigerous hairs are completely cleaned off by the morning after larval release. (A) An ovigerous seta just after larval release. The empty egg cases, funiculi, and investing coats are still attached to the ovigerous hairs. (B) An ovigerous seta (os) on the next morning (about 10 h) after the larval release.

mobile larvae (Rittschof *et al.*, 1990). In *S. haematocheir*, ovigerous females treated with hatch water certainly liberated premature embryos (Fig. 3B–E'); but in no case was the funiculus dissolved and detached from the investment coat (see Fig. 4A). As shown in Figures 4B, 5A, and 5B, the *coat* did slip off the ovigerous hairs. So what is liberated from *S. haematocheir* females is *masses* of premature embryos, and no individual free embryos.

The next question concerns the other functions of the incubation-disrupting substance. Because this factor is released from the egg case at the time of hatching, the factor must also be more directly involved in hatching. Recently, De Vries and Forward (1991) indicated that a proteolytic activity is released outside of the egg membrane in association with the hatching of estuarine crabs; the assay was based on the proteolysis of casein. But how, exactly, such an enzyme contributes to hatching remains obscure. As shown in Figures 4A, 5A, and 5B, the incubation-disrupting substance dissolved neither the outer egg membrane nor the funiculi. Neither was the investment coat itself dissolved (Fig. 4B). But these negative results might have been due to the relatively coarse scanning microscopy of the egg membrane. In any event, the possibility that the incubation-disrupting substance also functions as a hatching enzyme remains to be explored.

Acknowledgments

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